MOLECULAR AND CELLULAR BIOLOGY, Nov. 1996, p. 6427–6435 Vol. 16, No. 11
0270-7306/96/$04.00+0

Requirement for Phosphatidylinositol 3-Kinase in Epidermal Growth Factor-Induced AP-1 Transactivation and Transformation in JB6 P^+ Cells

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Received 15 July 1996/Accepted 6 August 1996

Phosphatidylinositol 3-kinase (PI 3-kinase) plays a role in a variety of biological processes, including regulation of gene expression, cell growth, and differentiation. However, little is known about its role in the cytoplasmic events involved in epidermal growth factor (EGF)-induced transduction of signals to the transcriptional machinery of the nucleus and in EGF-induced cell transformation. In this study, we examined whether PI 3-kinase is a mediator for the activation of AP-1 and neoplastic transformation by EGF in the murine epidermal cell line JB6. The results showed the following. (i) EGF not only induced a high level of PI 3-kinase activity by itself but also enhanced insulin-induced PI 3-kinase activity in JB6 P^+ cells, the EGF-induced PI-3 kinase activity could be blocked by constitutive overexpression of a dominant negative P85 subunit of PI 3-kinase (ΔP85), and insulin could markedly promote EGF-induced AP-1 activity in a dose-dependent manner in JB6 P^+ cells as well as promote EGF-induced JB6 P^+ cell transformation. (ii) Inhibition of PI-3 kinase with wortmannin or LY294002 markedly decreased the AP-1 activity induced by insulin, EGF, or EGF and insulin in a dose-dependent manner, while wortmannin did not block UVB-induced AP-1 activity. (iii) AP-1 activation by insulin, EGF, or EGF and insulin could be completely inhibited by overexpression of ΔP85 in all the dose and time courses studied. (iv) Inhibitors of PI 3-kinase (wortmannin and LY294002) and stable overexpression of ΔP85 inhibited EGF-induced transformation but had no significant inhibitory effect on cell proliferation induced by EGF or EGF and insulin. These results demonstrate for the first time that PI 3-kinase appears to be required for EGF- or insulin-induced AP-1 transactivation and cell transformation but not cell proliferation in JB6 cells.

Phosphatidylinositol 3-kinase (PI 3-kinase) is an important enzyme associated with a variety of receptors or protein-tyrosine kinases and acts as a direct biochemical link between a novel phosphatidylinositol pathway and a number of receptor proteins, including the receptors for insulin or platelet-derived growth factor. This enzyme is a heterodimer of a 110-kDa (P110) catalytic subunit and an 85-kDa (P85) regulatory subunit (5). It can phosphorylate phosphatidylinositol (Ptdins), Ptdins(4) phosphate [Ptdins(4)P], or Ptdins(4,5) bisphosphate [Ptdins(4,5)P2] to produce Ptdins(3)P, Ptdins(3,4)P2, or Ptdins (3,4,5) trisphosphate [Ptdins(3,4,5)P3], respectively (2, 5, 52). Insulin or growth factor stimulation of the associated tyrosine kinase results in phosphorylation of the P85 subunit of PI 3-kinase. This phosphorylation is important for activation of PI 3-kinase (6).

Several studies suggested that the PI 3-kinase products Ptdins(3,4)P2 and Ptdins(3,4,5)P3 are important regulators of cell proliferation (4, 21, 23). The introduction of the N-terminal SH_2 domain of the P85 subunit of PI 3-kinase into cells abrogates insulin- or insulin-like growth factor I (IGF-I)-stimulated DNA synthesis and prevents insulin stimulation of c-*fos* protein expression (21, 23). Other studies have shown that Ptdins(3,4)P2 and Ptdins(3,4,5)P3 levels are elevated in cells transformed by v-*abl*, v-*src*, and polyomavirus middle T, and decreased levels of these lipids correlate with impaired cell transformation by mutated forms of these oncogenes (4, 16, 47). Recently, it was reported that insulin could activate the Ras–Raf–mitogen-activated protein (MAP) kinase pathway by interacting with and activating its receptors (45, 53). Hu et al. suggest that activation of this Ras-MAP kinase pathway is critical for the effect of insulin on mitogenesis and c-*fos* expression (21). Others found that neither insulin nor phorbol ester regulation of phosphoenolpyruvate carboxykinase gene expression requires activation of the Ras-MAP kinase pathway but that PI 3-kinase is required in this event (17, 54). In contrast, Sakaue et al. demonstrated that neither the Ras-MAP kinase cascade nor PI 3-kinase may be required for insulin-stimulated glycogen synthase activation in CHO cell lines (32). It is well known that epidermal growth factor (EGF) can induce a high level of AP-1 activity and cell transformation as well as PI 3-kinase activity. Cell transformation is a complex process and much different from cell mitogenesis; in some cases, these two events are dissociated (7, 20, 29, 41, 51). Currently, little is known regarding whether PI 3-kinase and its products are involved in EGF-induced signal transduction to the transcriptional machinery of the nucleus and cell transformation. We used the well-characterized mouse epidermal JB6 P^+ (tumor promotion-sensitive) cells and addressed a novel function of PI 3-kinase in EGF-induced AP-1 transactivation and cell transformation.

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MATERIALS AND METHODS

Plasmids and reagents. The AP-1 luciferase reporter plasmid (Col-Luc) and CMV-neu marker vector plasmid were constructed as previously reported (12); the bovine PI 3-kinase P85 subunit mutant plasmid (ΔP85) and vector plasmid SRα (18) were generous gifts from Masato Kasuga, School of Medicine, Kobe University, Kobe, Japan. Agarose conjugated with monoclonal antiphosphotyrosine antibody Py20 was obtained from Santa Cruz, fetal bovine serum (FBS) was from GIBCO, Lipofectamine was from GIBCO BRL, Eagle's minimal essential medium (MEM) and wortmannin were from Calbiochem, LY294002 was from Biomol, EGF was from Collaborative Research, insulin was from Sigma,

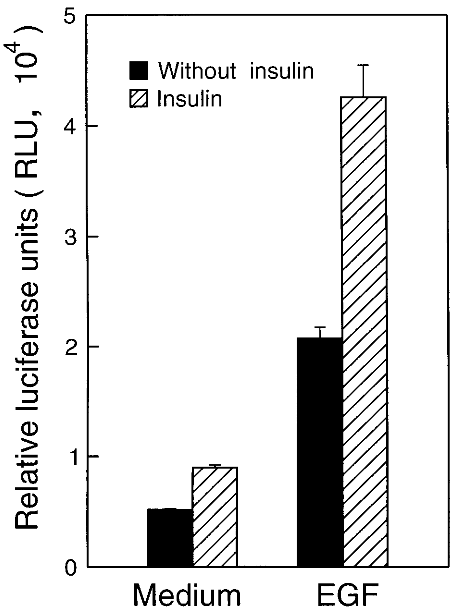

FIG. 1. Insulin induces AP-1 transactivation and enhances EGF-induced AP-1 activity. JB6 Cl 41-19 cells were exposed to EGF (10 ng/ml) or were not exposed, with or without insulin (2.5 μg/ml), at 37°C in 5% CO_2 for 24 h. The AP-1 activity was determined by luciferase activity assay as described in Materials and Methods.

luciferase assay substrate was from Promega, and [γ-^{32}P]ATP was from DuPont NEN.

Cell culture. The JB6 P$^+$ mouse epidermal cell line Cl 41, the stable AP-1 luciferase reporter plasmid-transfected mouse epidermal JB6 P$^+$ cell line Cl 41-19, and JB6 transformed cell line A33 (25, 27) were cultured in monolayers at 37°C with 5% CO_2 in MEM containing 5% fetal calf serum, 2 mM L-glutamine, and 25 μg of gentamicin per ml.

Generation of stable cotransfectants with the AP-1 reporter and dominant-negative PI 3-kinase mutant. JB6 P$^+$ Cl 41 cells were cultured in a six-well plate until they reach 85 to 90% confluence. We used 2 μg of AP-1 luciferase reporter plasmid and 0.3 μg of CMV-neo vector with 6 μg of dominant-negative mutant of PI 3-kinase P85 subunit plasmid ΔP85 or vector SRα control plasmid DNA and 15 μl of Lipofectamine reagent to transfect each well in the absence of serum. After 10 to 12 h, the medium was replaced by 5% FBS–MEM. Approximately 30 to 36 h after the beginning of the transfection, the cells were trypsinized and 75-ml culture flasks were seeded with cell suspensions which were then cultured for 24 to 28 days with G418 (300 μg/ml) selection. Stable transfectants were screened by assaying the luciferase activity and Western blotting (immunoblotting) with rabbit polyclonal immunoglobulin G against human PI 3-kinase P85 antibody. Stably transfected ΔP85 mass1, ΔP85 mass2, and AP-1 mass1 cells were cultured in G418-free MEM for at least two passages before each experiment.

Assay for AP-1 activity. Confluent monolayers of JB6 Cl 41-19, A33, ΔP85 mass1, ΔP85 mass2, or AP-1 mass1 cells were trypsinized, and 5×10^3 viable cells suspended in 100 μl of 5% FBS–MEM were added to each well of a 96-well plate. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. Twelve to twenty-four hours later, the cells were starved by being cultured in 0.1% FBS–MEM for 12 h prior to exposure to EGF or insulin. The cells were exposed to EGF, insulin, and EGF plus insulin for AP-1 induction with or without various concentrations of wortmannin or LY294002 for 24 h. The cells were extracted with lysis buffer, and luciferase activity was measured with a luminometer (monolight 2010). The results are expressed as relative AP-1 activity or relative luciferase units (3, 41).

PI 3-kinase assay. PI 3-kinase activity was assayed as previously described (15). In brief, JB6 cells (Cl 41, ΔP85 mass1 and ΔP85 mass2, or AP-1 mass1) were cultured in monolayers in 100-mm-diameter plates; then the cells were incubated in serum-free MEM for 3 to 4 h at 37°C, 10 ng of EGF per ml with or without wortmannin was added, and after 20 min at 37°C, 2.5 μg of insulin per ml was added. After 10 min at 37°C, the cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed in 400 μl of lysis buffer per plate (20 mM Tris [pH 8], 137 nM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40, 1 nM dithiothreitol, 0.4 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride). The lysates were centrifuged, and the supernatants were incubated with 40 μl of agarose beads previously conjugated with the monoclonal antiphosphotyrosine antibody Py20 overnight at 4°C. The beads were washed twice with each of the following buffers: (i) PBS with 1% Nonidet P-40 and 1 mM dithiothreitol, (ii) 0.1 M Tris (pH 7.6)–0.5 M LiCl–1 mM dithiothreitol, and (iii) 10 mM Tris (pH 7.6)–0.1 M NaCl–1 mM dithiothreitol. The beads were incubated for 5 min on ice in 20 μl of the third buffer, and then 20 μl of 0.5-mg/ml Ptdins which had been previously sonicated in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.6)–1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)–1 mM NaH_2PO_4 was added. After 5 min at room temperature, 10 μl of the reaction buffer (50 mM $MgCl_2$–100 mM HEPES [pH 7.6]–250 μM ATP containing 5 μCi of [γ-^{32}P]ATP) was added, and the beads were incubated for an additional 5 min. The reactions were stopped by the addition of 15 μl of 4 N HCl and 130 μl of chloroform-methanol (1:1). After 30 s of vortexing, 30 μl from the phospholipid-containing chloroform phase was spotted onto thin-layer chromatography plates which had previously been coated with 1.3% potassium oxalate–2 mM EDTA in H_2O-methanol (3:2) and baked at 110°C for at least 3 h before the spotting. The plates were placed in tanks containing chloroform-methanol-NH_4OH-H_2O (600:470:20:113) for 40 to 50 min, until the solvent reached the top of the plates. The plates were dried at room temperature and autoradiographed, and the phosphatidylinositol 3-phosphate spots were scraped off and counted.

Southern blot. Isolation of genomic DNA and Southern hybridization were performed according to established methods (33). Briefly, 15 μg of genomic DNA from each AP-1 reporter-transfected clonal line or the parental cell line Cl 41 was digested with restriction endonucleases *Bam*HI and *Eco*RV, and DNAs were separated by 0.9% gel electrophoresis. The DNA was denatured and transferred onto nucleic acid transfer membranes (Hybond-N$^+$). The filter was hybridized with the luciferase gene *Bam*HI-*Eco*RV DNA fragments of PG12-luciferase vector (Promega), which were labeled with [^{32}P]dCTP by using a random primer labeling kit (Amersham). Prehybridization, hybridization, and blocking were performed in the presence of 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C. Selective hybridization of luciferase sequences was achieved by washing the filters in 0.1× SSC containing 0.5% sodium dodecyl sulfate at 68°C. The hybridization blot was visualized by being exposed to X-ray film.

Anchorage-independent transformation assay. Inhibition by wortmannin or LY294002 of EGF- or EGF-plus-insulin-induced cell transformation was investigated with JB6 P$^+$ cells. A total of 10^4 cells were exposed to EGF or EGF plus insulin with or without wortmannin or LY294002 in 1 ml of 0.33% basal medium Eagle (BME) agar containing 10% FBS laid over 3.5 ml of 0.5% BME agar containing 10% FBS in each well of a 60-mm-diameter dish. The cultures were maintained in a 37°C, 5% CO_2 incubator for 14 to 16 days, and the cell colonies were scored by previously described methods (7). The efficiency of wortmannin or LY294002 in inhibiting transformation of JB6 cells was presented as a percentage of inhibition of cell transformation.

Assay for cell proliferation. Cell proliferation was determined by [^{3}H]thymidine incorporation assay. For the study of the influence of wortmannin or

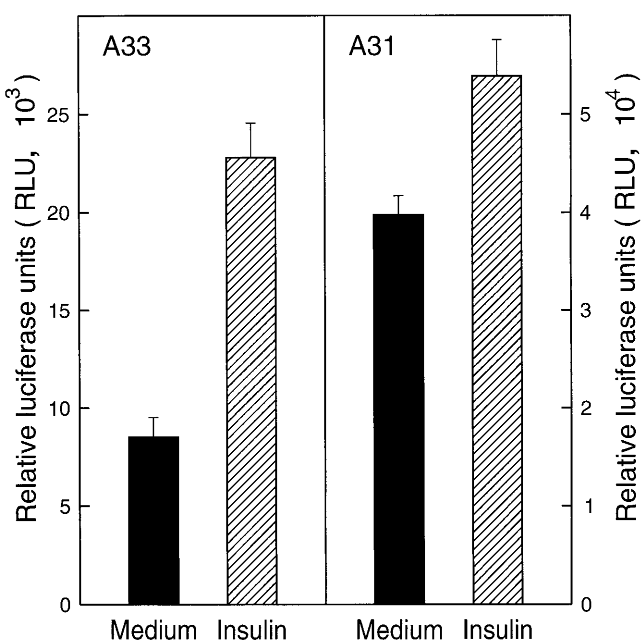

FIG. 2. Influence of insulin on AP-1 activity of transformed JB6 cells. A31 or A33 cells were untreated or treated with insulin (2.5 μg/ml) at 37°C in 5% CO_2 for 24 h. The AP-1 activity was measured and presented as described for Fig. 1 as well as in Materials and Methods.

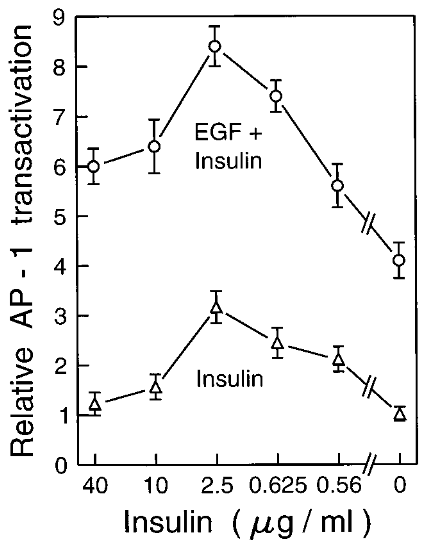

FIG. 3. Enhancement of EGF-induced AP-1 activity in JB6 P+ cells by insulin. JB6 Cl 41-19 cells were exposed to EGF (10 ng/ml) or were unexposed, with or without different concentrations of insulin, at 37°C in 5% CO_2 for 24 h. The AP-1 activity was measured by luciferase activity assay as described in Materials and Methods. The results are presented as fractions of the luciferase activity in control cells.

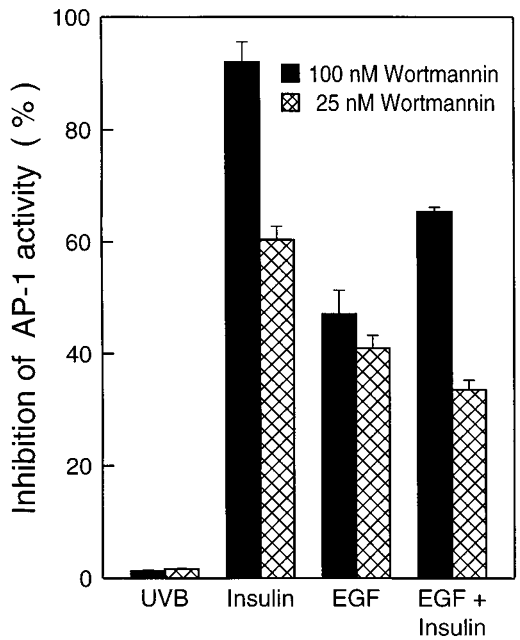

FIG. 4. Inhibition of AP-1 activity by wortmannin. JB6 Cl 41-19 cells were first treated with different concentrations of wortmannin (25 or 100 nM) for 30 min. The cells were then exposed to UVB (2 kJ/m^2), insulin (2.5 μg/ml), EGF (10 ng/ml), or insulin (2.5 μg/ml) plus EGF (10 ng/ml). After 24-h culture at 37°C in 5% CO_2, the AP-1 activity was measured and presented as described in Materials and Methods.

LY294002 on cell proliferation, 5×10^3 JB6 Cl 41-19 cells were seeded in 96-well microtiter plates in the presence of wortmannin or LY294002. For the investigation of the effect of overexpression of ΔP85 on cell proliferation, 96-well microtiter plates were seeded with 5×10^3 AP-1 mass1, ΔP85 mass1, or ΔP85 mass2 cells. After 36 h of culture, [^{3}H]thymidine (0.5 μCi per well) was added to each well. The cells were harvested 12 h later, and incorporation of [^{3}H]thymidine was detected with a liquid scintillation counter. The results were presented as counts per minute and are the averages and standard deviations for assays of the triplicate wells.

RESULTS

Insulin induces AP-1 transactivation and enhances EGF-induced AP-1 activity. To investigate whether insulin could induce AP-1 activity or promote EGF-induced AP-1 activity in JB6 cells, we exposed JB6 P+ cells to insulin, EGF, or EGF plus insulin. The results showed that insulin could markedly induce AP-1 activity and increase EGF-induced AP-1 activity in JB6 P+ cells (Fig. 1) and JB6 transformed cell lines A31 and A33 (Fig. 2) with a 0.1% FBS medium culture system. The induction of AP-1 activity and increase of EGF-induced AP-1 activity by insulin occur in a dose-dependent manner (Fig. 3).

Inhibition of EGF-induced AP-1 activity in JB6 P+ cells by wortmannin. Since the above results show that insulin could stimulate AP-1 activity and enhance EGF-induced AP-1 activity, and since insulin is an activator of PI 3-kinase, we hypothesize that PI 3-kinase may be involved in insulin- or EGF-induced AP-1 transactivation. To test this idea, we incubated JB6 P+ cells with wortmannin, a PI 3-kinase inhibitor which acts by covalently binding to P110 of PI 3-kinase, for 30 min prior to the addition of stimulators. The results showed that a nontoxic concentration of wortmannin (100 nM) inhibited 90.0, 47.1, or 65.4% of AP-1 activity induced by insulin, EGF, or EGF plus insulin, respectively, while no significant inhibition of UVB-induced AP-1 activity by wortmannin was seen (Fig. 4).

EGF induces PI 3-kinase activity and enhances insulin-induced PI 3-kinase activity. To investigate whether EGF can induce PI 3-kinase activity, we analyzed PI 3-kinase activity in JB6 P+ cells stimulated by EGF. As shown in Fig. 5, EGF not only induced a high level of PI 3-kinase activity, but also promoted insulin-induced PI 3-kinase activity. Enhancement of insulin-induced PI 3-kinase activity by EGF could be blocked by wortmannin (Fig. 5).

Inhibition of EGF-induced AP-1 activity in JB6 P+ cells by LY294002. Because wortmannin was recently reported to inhibit not only PI 3-kinase but also phospholipase A2 (PLA_2) activity induced in 3T3 cells by bombesin at a similar concentration (9), whether inhibition of AP-1 activity by wortmannin is targeted to PI 3-kinase requires further evidence. We therefore used another PI 3-kinase inhibitor, LY294002, to determine the role of PI 3-kinase in AP-1 transactivation induced by insulin or EGF. Unlike wortmannin, LY294002 inhibits PI 3-kinase by competing with ATP for its substrate binding site (48). The results showed that LY294002 inhibits AP-1 activity induced by various agents in a dose-dependent manner (Fig. 6).

Overexpression of the dominant-negative PI 3-kinase P85α blocks insulin- or EGF-induced AP-1 activity. Since significant inhibition of insulin- or EGF-induced AP-1 activity was achieved by using both PI 3-kinase inhibitors, wortmannin and LY294002, PI 3-kinase may play a critical role in EGF- or insulin-induced AP-1 activation. The dominant-negative mutant of PI 3-kinase, ΔP85, has been shown to be a specific inhibitor of PI 3-kinase in CHO cells (18). To specifically block PI 3-kinase and test the role of PI 3-kinase in EGF-induced AP-1 activation in JB6 cells, the dominant-negative mutant of the PI 3-kinase regulatory subunit P85 plasmid and the AP-1 reporter were cotransfected into JB6 cells by using a Lipofectamine kit. Three stable mass cultures, two (ΔP85 mass1 and ΔP85 mass2) from cotransfection with the AP-1 reporter and SRα ΔP85 plasmids and one (AP-1 mass1) from cotransfection with the AP-1 reporter and vector SRα, were estab-

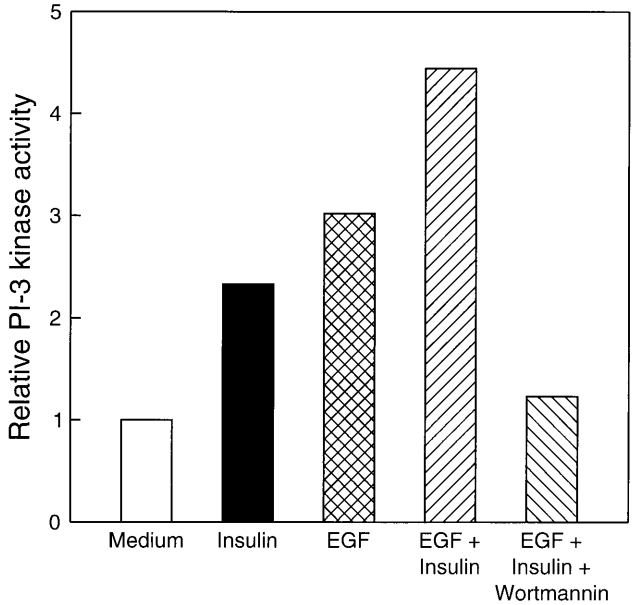

FIG. 5. EGF induces PI 3-kinase activity and enhances insulin-induced PI 3-kinase activity. JB6 Cl 41 cells were treated with medium or EGF (10 ng/ml) with or without wortmannin for 20 min at 37°C in 5% CO_2. The cells were then exposed to insulin (2.5 μg/ml) for another 10 min. The cells were harvested and PI 3-kinase activity was measured as described in Materials and Methods. The results are presented as the fraction of PI 3-kinase activity in control cells.

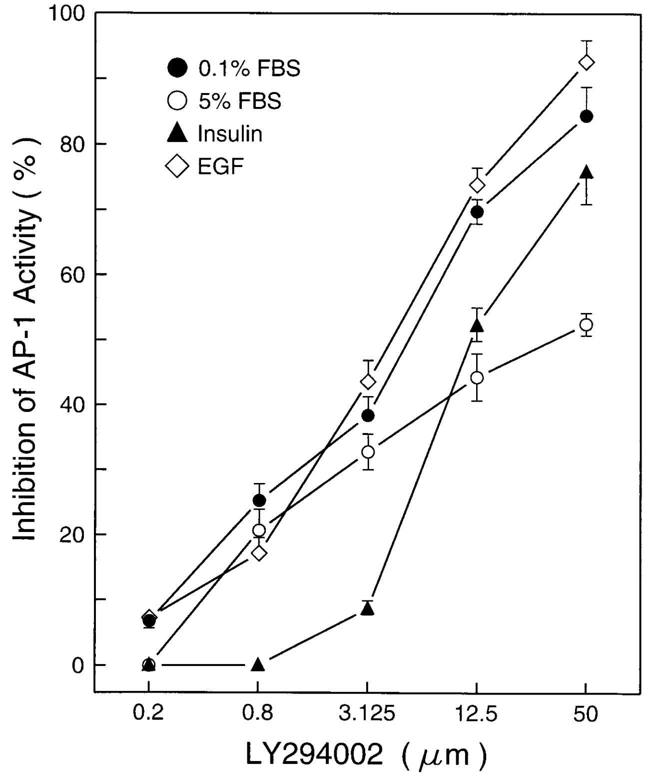

FIG. 6. Inhibition of AP-1 activity by LY294002. JB6 Cl 41-19 cells were first treated with different concentrations of LY294002 (from 0.2 to 50 μM) for 30 min. The cells were then exposed to the indicated inducers. After 24-h culture at 37°C in 5% CO_2, the AP-1 activity was measured and presented as described in Materials and Methods.

lished by G418 selection. Expression of dominant-negative PI 3-kinase protein in ΔP85 mass1 and ΔP85 mass2 was detected in comparison with that in AP-1 mass1 cells by using rabbit polyclonal immunoglobulin G against the PI 3-kinase P85 subunit for immunoprecipitation and Western blotting (data not shown). To determine whether overexpression of dominant-negative PI 3-kinase protein blocks PI 3-kinase activity, we also tested the PI 3-kinase activity in ΔP85 mass1 and mass2 cells induced by insulin, EGF, or EGF plus insulin. The results shown that the PI 3-kinase activity induced by insulin, EGF, or EGF plus insulin was totally blocked by expression of dominant-negative PI 3-kinase protein (Fig. 7). Furthermore, overexpression of dominant-negative PI 3-kinase protein in ΔP85 mass1 and ΔP85 mass2 cells inhibited basal AP-1 activity (Table 1) and blocked insulin- or EGF-stimulated AP-1 activity completely compared with that in AP-1 mass1 cells in all the time and dose courses studied (Fig. 8A and Fig. 9). To be sure that the blocking of AP-1 activity by overexpression of ΔP85 was not due to different copies of the AP-1 luciferase reporter gene among AP-1 mass1, ΔP85 mass1, and ΔP85 mass2 cells, we used Southern blotting to analyze the AP-1 reporter gene in the genomic DNAs of these stable transfectants. The results indicated that similar copies of the reporter gene in the genomic DNAs were observed in these stable transfectants (Fig. 8B).

Enhancement by insulin of EGF-induced JB6 P^+ cell transformation. Since our previous results and other studies demonstrated that induced AP-1 activity is important and required for cell transformation, we tested whether insulin could induce transformation or promote EGF-induced transformation. The results showed that insulin could not induce JB6 P^+ cell transformation alone; however, it markedly increased the EGF-induced JB6 P^+ cell transformation rate (Fig. 10).

Inhibition by LY294002 of JB6 P^+ cell transformation induced by EGF and EGF plus insulin. As shown in Fig. 11, LY294002 inhibited not only EGF-plus-insulin-induced JB6 P^+ cell transformation but also EGF-induced JB6 P^+ cell transformation. The inhibition by wortmannin of EGF- or EGF-plus-insulin-induced cell transformation was consistent with data obtained by using LY294002 (data not shown). This inhibition is in a dose range similar to that observed for the inhibition of PI 3-kinase activity and the inhibition of AP-1 transactivation.

Blocking of JB6 cell transformation by overexpression of ΔP85 protein. We further explored whether the transfectants expressing the dominant-negative PI 3-kinase mutant could repress EGF-induced transformation. Figure 12 summarizes the results of these studies. The EGF-induced transformation in two stable mass transfectants, ΔP85 mass1 and ΔP85 mass2, was almost totally blocked, while the AP-1 mass1 cells showed a high frequency of transformation with exposure to EGF.

No inhibition of cell mitogenesis by wortmannin, LY294002, or overexpression of ΔP85. Previous studies showed that PI 3-kinase plays an important role in certain types of cell mitogenesis (4, 21, 23). To investigate the role of PI 3-kinase in mitogenesis of JB6 cells, we tested the influence of wortmannin, LY294002, and overexpression of ΔP85 on JB6 cell proliferation. The results indicated that neither PI 3-kinase pharmacologic inhibitors (wortmannin and LY294002) nor a molecular inhibitor (overexpression of ΔP85) significantly inhibited JB6 cell proliferation (Fig. 13). Figure 13 also shows that insulin could not enhance the EGF-stimulated JB6 cell proliferation.

DISCUSSION

The data presented in this paper demonstrate a novel function of PI 3-kinase in EGF-induced AP-1 transactivation and

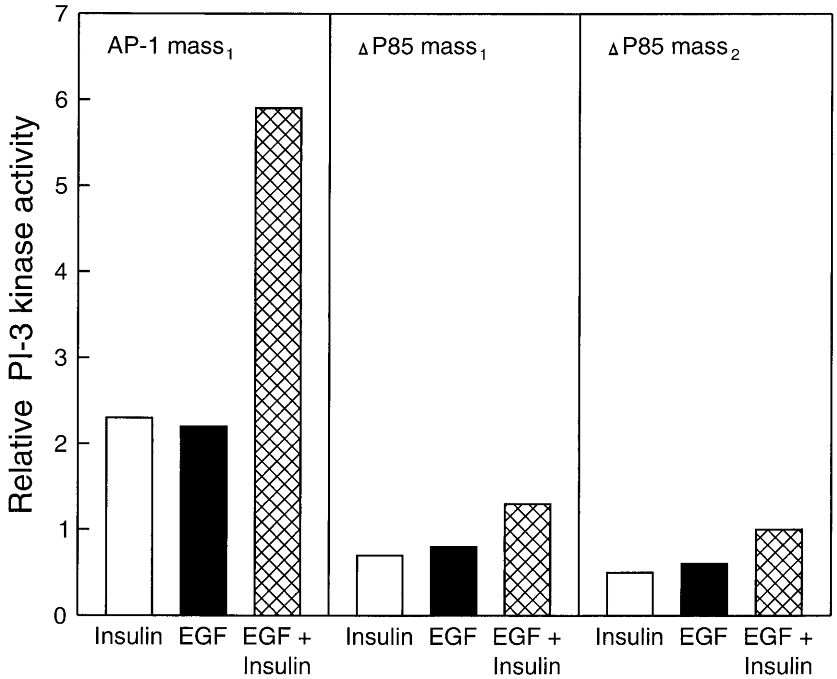

FIG. 7. Inhibition of PI 3-kinase activity by overexpression of a dominant-negative PI 3-kinase mutant. Cells (ΔP85 mass1, ΔP85 mass2, or AP-1 mass1) were untreated or treated with EGF (10 ng/ml) for 20 min at 37°C in 5% CO_2. They were then exposed to insulin (2.5 μg/ml) for another 10 min. The cells were harvested and PI 3-kinase activity was measured as described in Materials and Methods.

transformation. First, insulin, a strong PI 3-kinase activator, enhanced EGF-induced AP-1 transactivation and transformation in JB6 P^+ cells. Second, EGF induced PI 3-kinase and promoted insulin-induced PI 3-kinase in the JB6 cell system, and wortmannin or overexpression of ΔP85 protein inhibited EGF- and insulin-induced PI 3-kinase. Finally, we used both pharmacologic PI 3-kinase inhibitors, wortmannin and LY294002, and a molecular inhibitor, a dominant-negative mutant of PI 3-kinase, to test the hypothesis that PI 3-kinase is a mediator for EGF-induced AP-1 activation and transformation in JB6 cells. Inhibition of the PI 3-kinase with wortmannin or LY294002 decreased the AP-1 activity induced by insulin, EGF, or EGF plus insulin and inhibited JB6 P^+ cell transformation induced by EGF. Constitutive overexpression of the dominant-negative PI 3-kinase P85 mutants completely blocked insulin- or EGF-induced AP-1 transactivation and EGF-induced cell transformation. The extent of inhibition of PI 3-kinase by wortmannin or overexpression of ΔP85 is in agreement with the extent of inhibition of AP-1 and cell transformation.

Cell transformation is a complex process which is different from cell mitogenesis and involves a wide array of signaling events, such as the MAP kinase signal cascade and other signal pathway (7, 20, 28, 29, 41, 50, 51). In some cases, cell transformation is dissociated from mitogenesis (7, 20, 41). Previous studies have shown that PI 3-kinase plays a crucial role in cell proliferation and c-*fos* gene expression (21, 23). The introduction of the N-terminal SH_2 domain of the P85 subunit of the PI 3-kinase into cells abrogated insulin- or IGF-I-stimulated DNA synthesis and prevented c-*fos* protein expression (23). The microinjection of a dominant-negative p21^{ras} mutant or anti-*ras* antibody inhibited insulin-induced DNA synthesis (23). A constitutively activated mutant P110 induced transcription from the *fos* promoter; coexpression of dominant-negative Ras blocked this response (21). It has been reported that the presence of IGF-I receptor (IGF-IR) is an obligatory requirement for the establishment and maintenance of the tumor phenotype (3, 8, 29, 41). Cells derived from mouse embryos with a targeted disruption of the IGF-IR gene (R^- cells) cannot be transformed by simian virus 40 T antigen or by an activated and overexpressed Ha-Ras, or even by a combination of both, all of which transform very efficiently the corresponding wild-type cells or other 3T3-like cells (35, 36). If a plasmid expressing a wild-type human IGF-IR cDNA is stably transfected into R^- cells, the cells could be transformed by simian virus 40 T antigen. This indicated that the defect in transformability is due specifically to the lack of IGF-IR (15, 36, 46). Substantial evidence that PI 3-kinase is a critical component of signaling pathways used by the cell surface receptors for a variety of mammalian growth factors or other stimulators has been reported (2, 4, 14, 19, 39, 40, 44, 45), especially insulin receptor (IR) and IGF-IR. Different groups using different models have pointed out the crucial importance of AP-1 activity in transformation and carcinogenesis (1, 10, 11, 13, 22). Previous results from studies by us and others have shown that AP-1 activation is required for tumor promotion in the JB6 cell model (11, 13, 22). High basal levels of AP-1 activity appear to be important for the maintenance of tumor phenotypes in the transformed cell line RT101 (11, 13). The physiological prod-

TABLE 1. Basal level of AP-1 activity in stable transfectants[a]

Cell type	Plasmids	AP-1 luciferase units
Cl 41		150 ± 28
AP-1 mass1	Col-Luc + SRα	14,287 ± 587
ΔP85 mass1	Col-Luc + SRα ΔP85	1,867 ± 135
ΔP85 mass2	Col-Luc + SRα ΔP85	1,601 ± 184

[a] Six-well plates were seeded with 5×10^4 cells in 5% FBS–MEM; after culture in a 37°C incubator for 12 to 24 h, the cells were extracted and luciferase activity was assayed as described in Materials and Methods.

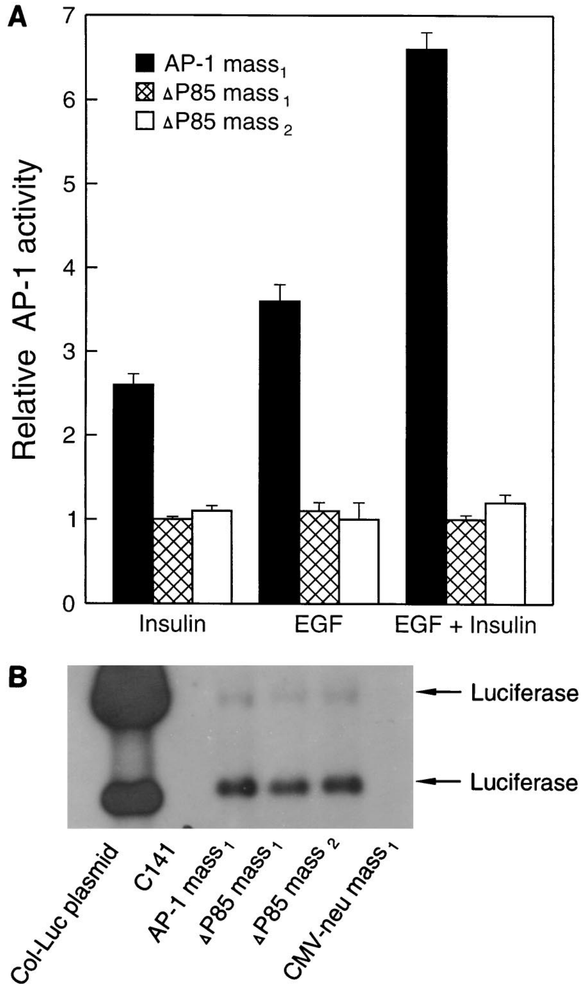

FIG. 8. Inhibition of insulin-, EGF-, or EGF-plus-insulin-induced AP-1 activity by overexpression of a dominant-negative PI 3 kinase mutant (A) and stable presence of the luciferase gene in reporter stable transfectants (B). (A) Cells (ΔP85 mass1, ΔP85 mass2, or AP-1 mass1) were exposed to insulin (2.5 μg/ml), EGF (10 ng/ml), or insulin (2.5 μg/ml) plus EGF (10 ng/ml) or were left unexposed. After 24-h culture at 37°C in 5% CO_2, the AP-1 activity was measured and presented as described in Materials and Methods. (B) Genomic DNA from each cell line was digested with restriction enzymes *Bam*HI and *Eco*RV. The digestion products were separated by 0.9% agarose electrophoresis and transfected onto a nucleic acid transfer membrane. The filter was hybridized with ^{32}P-labeled luciferase cDNA probes and visualized on X-ray film.

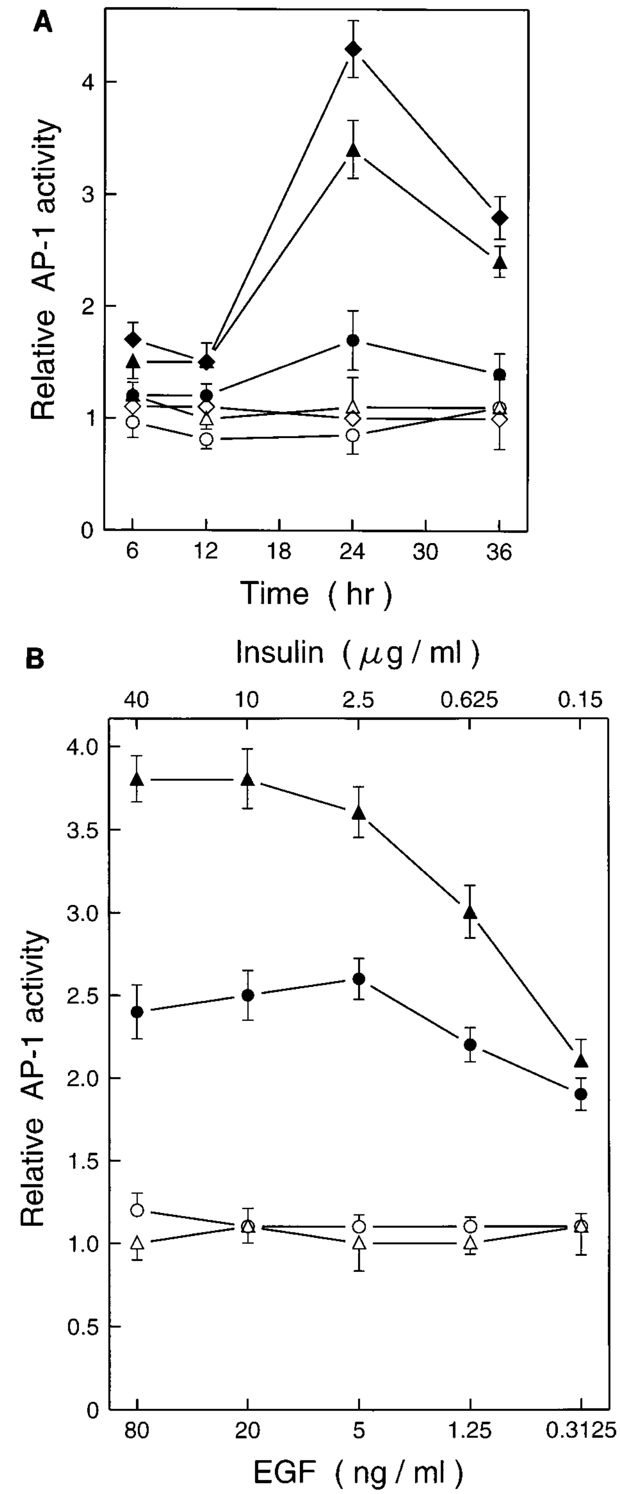

FIG. 9. Time course and dose response of inhibition of AP-1 activity by overexpression of a dominant-negative PI 3-kinase mutant. (A) For the time course study, ΔP85 mass2 (open symbols) or AP-1 mass1 (closed symbols) cells were exposed to 2.5 μg of insulin per ml (circles), 10 ng of EGF per ml (triangles), or 2.5 μg of insulin per ml plus 10 ng of EGF per ml (diamonds) or were left unexposed, and the AP-1 activity was determined at different times as indicated. (B) For the dose-response study, cells were treated with the indicated doses of insulin (circles) or EGF (triangles) for 24 h. The AP-1 activity was measured and presented as described in Materials and Methods.

uct of PI 3-kinase is the highly polar membrane lipid Ptdins(3, 4, 5)P3 (4, 14, 19, 39, 40, 44). This lipid has been postulated to act as a second messenger in the cell (4, 14, 40). It has been reported that EGF can induce a 10-fold increase of 3′-phosphorylated phosphoinositides in PC12 cells (30), but little is known about these lipid functions.

Since PI 3-kinase mediates IGF-IR-induced signaling and IGF-IR is required for transformation, we addressed the importance of PI 3-kinase activity for EGF-induced AP-1 transactivation and cell transformation in JB6 cells. First, we treated EGF-induced cells with insulin, a very effective PI 3-kinase stimulator, and EGF-induced AP-1 activity and transformation, but not cell proliferation, were increased significantly. Insulin is able to bind to both IR and IGF-IR (34, 42), but the affinities of these two receptors for insulin are different. The affinity of IR for insulin is at least 100 times higher than that of IGF-IR (34). The optimal concentration of insulin on AP-1

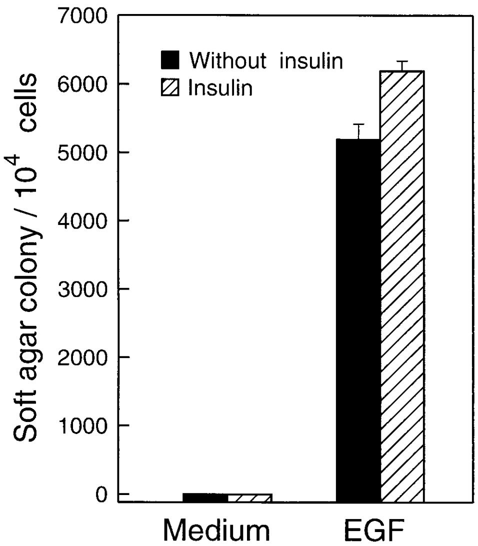

FIG. 10. Enhancement by insulin of EGF-induced JB6 P$^+$ cell transformation. A total of 10^4 JB6 Cl 41 cells were left unexposed or were exposed simultaneously to insulin (2.5 μg/ml), EGF (10 ng/ml), or EGF (10 ng/ml) plus insulin in 0.33% BME agar containing 10% FBS over 0.5% BME agar medium containing 10% FBS. Cell colonies were scored after 14 days of incubation at 37°C in 5% CO_2.

activation and cell transformation used in this study is 2.5 μg/ml. At this concentration, insulin may bind and interact with both IR and IGF-IR. With whichever receptor the insulin binds and interacts, after IR and IGF-IR activation, the tyrosine-phosphorylated form of insulin receptor substrate 1 binds the P85 subunit of PI 3-kinase, and this interaction results in the activation of the PI 3-kinase. Therefore, the finding

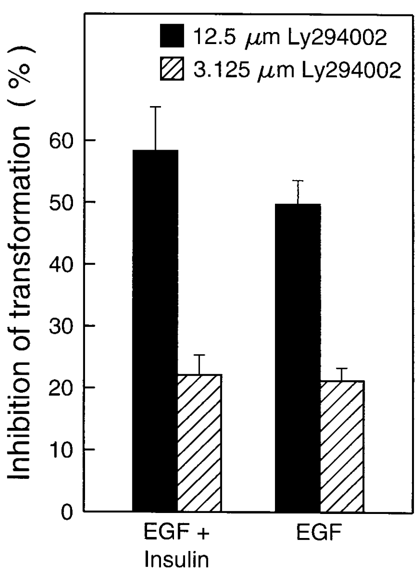

FIG. 11. Inhibition by LY294002 of JB6 P$^+$ cell transformation induced by EGF alone or EGF plus insulin. A total of 10^4 JB6 Cl 41 cells were left unexposed or were exposed simultaneously to EGF (10 ng/ml) or EGF (10 ng/ml) plus insulin (2.5 μg/ml) with or without LY294002 (at the indicated concentrations) in 0.33% BME agar containing 10% FBS over 0.5% BME agar containing 10% FBS. Cell colonies were scored after 14 days of incubation at 37°C in 5% CO_2. The inhibition of AP-1 cell transformation induced by EGF or EGF plus insulin is expressed as described in Materials and Methods.

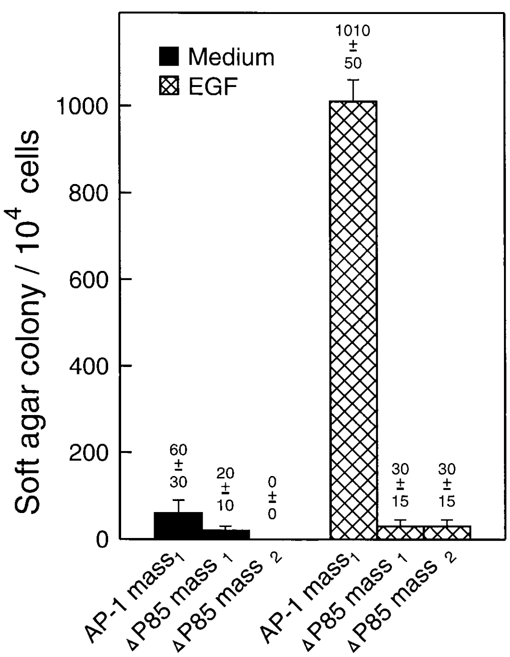

FIG. 12. Inhibition of JB6 P$^+$ cell transformation induced by EGF by overexpression of a dominant-negative PI 3-kinase mutant. A total of 10^4 ΔP85 mass1, ΔP85 mass2, or AP-1 mass1 cells were left unexposed or were exposed to EGF (10 ng/ml) in 0.33% BME agar containing 10% FBS over 0.5% BME agar containing 10% FBS. Cell colonies were scored after 21 days of incubation at 37°C in 5% CO_2. The inhibition of cell transformation induced by EGF is expressed as described in Materials and Methods.

that insulin enhances EGF-induced AP-1 activation and cell transformation still supports the notion that PI 3-kinase plays an important role in EGF-induced AP-1 activation and cell transformation. Furthermore, in our study, we have used inhibitors of PI 3-kinase to test our hypothesis that PI 3-kinase is required for EGF-induced AP-1 activation and cell transformation. The first inhibitor is a fungal metabolite, wortmannin, which covalently binds to the catalytic subunit (P110) of mammalian PI 3-kinase and irreversibly inhibits the enzymatic activity at nanomolar concentrations (24, 43, 54). Since wortmannin was recently reported to inhibit PLA_2 activity at a concentration similar to that which inhibits PI 3-kinase activity (9), we also used another PI 3-kinase inhibitor, LY294002. Unlike wortmannin, LY294002 reversibly inhibits PI 3-kinase by competing with ATP for its substrate binding site and thus affects a very specific stimulating pathway (48). The other approach to testing our hypothesis that PI 3-kinase is required for EGF-induced AP-1 activation and cell transformation utilized stable introduction of a dominant-negative mutant of PI 3-kinase, ΔP85, into JB6 cells. This dominant-negative mutant has been shown to specifically block PI 3-kinase activity and its mediated signals and function (18, 32). Stable transfection of JB6 cells with ΔP85 blocks EGF- or insulin-induced PI 3-kinase activity, AP-1 activity, and cell transformation but does not significantly inhibit cell proliferation. All results from these experiments strongly support the hypothesis that PI 3-kinase is required for EGF-stimulated AP-1 activity and transformation, but not cell proliferation, in JB6 cells. This dissociation of cell mitogenesis and transformation is consistent with previous findings for JB6 cells (7, 11).

Insulin can elicit multiple biological responses by activating PI 3-kinase (26). These responses include promotion of cellu-

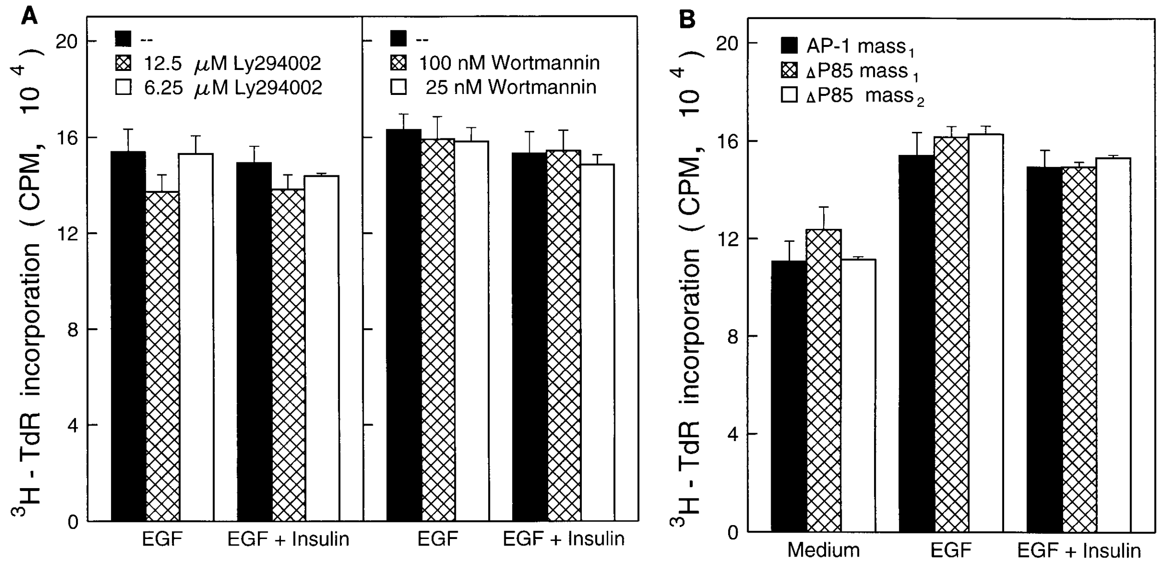

FIG. 13. Influence of overexpression of ΔP85 or PI 3-kinase inhibitors on JB6 cell proliferation. (A) To study the effect of wortmannin or LY294002 on cell proliferation, 96-well plates were seeded with 5×10^3 JB6 Cl 41-19 cells in the presence of wortmannin or LY294002. (B) To study the effect of overexpression of ΔP85 on cell mitogenesis, 96-well plates were seeded with 5×10^3 cells AP-1 mass1, ΔP85 mass1, or ΔP85 mass2 cells. After 36-h culturing in a 37°C incubator, [^{3}H]thymidine (^{3}H-TdR) was added to each well (0.5 μCi per well). The cells were harvested 12 h later, and incorporation of [^{3}H]thymidine was detected by using a liquid scintillation counter. Each bar indicates the average and standard deviation from triplicate assay wells.

lar growth and regulation of cell differentiation and metabolism in the target cells (26). Since PI 3-kinase could be coimmunoprecipitated with Ras (24, 54), it was suggested that this enzyme could be used as either an effector or a regulator of Ras (21, 30, 31, 37, 38). Ras is a GTP-binding protein which plays a central role in integrating extracellular signals for the regulation of cell function. A recent study suggested that MAP kinase plays a role in a negative-feedback mechanism which limits the extent of Ras activation (49). Stimulation of cells with insulin resulted in serine/threonine phosphorylation of SOS and dissociation of the Grb_2-SOS complex (49). In this study, as shown in Fig. 3, AP-1 activation or enhancement of EGF-induced AP-1 activation by insulin was observed in a dose-dependent manner only for insulin doses of <2.5 μg/ml. AP-1 activity induced by high concentrations of insulin appears to decrease. The interpretation of this phenomenon is that high concentrations of insulin may also induce a negative-feedback mechanism which negatively regulates AP-1 activation. It has been reported that EGF can induce a 10-fold increase of 3′-phosphorylated phosphoinositides in PC12 cells (30). Although the role of these lipids is unknown, it seems reasonable to assume that they play an important part in the regulation of cellular growth and other functions. Here, we present evidence that PI 3-kinase acts as a signal in EGF-induced AP-1 transactivation and neoplastic transformation, and we propose that EGF-induced AP-1 activation and transformation require PI 3-kinase-mediated signals. This is the first evidence that PI 3-kinase plays an important role in EGF-induced AP-1 activation as well as transformation, but not in EGF-induced cell proliferation, in JB6 cells.

ACKNOWLEDGMENTS

This work was supported by the Hormel Foundation.

We thank H. H. O. Schmid for critical reading, P. C. Schmid for help with PI 3-kinase activity analysis, Masato Kasuga for generous gifts of bovine PI 3-kinase P85 subunit mutant plasmid ΔP85 and vector plasmid SRα, and Carmen Perleberg for secretarial assistance.

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